REVIEW ARTICLE

Base pairing small RNAs and their roles in global regulatory networks

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Abstract

Bacteria use a range of RNA regulators collectively termed small RNAs (sRNAs) to help respond to changes in the environment. Many sRNAs regulate their target mRNAs through limited base-pairing interactions. Ongoing characterization of base-pairing sRNAs in bacteria has started to reveal how these sRNAs participate in global regulatory networks. These networks can be broken down into smaller regulatory circuits that have characteristic behaviors and functions. In this review, we describe the specific regulatory circuits that incorporate base-pairing sRNAs and the importance of each circuit in global regulation. Because most of these circuits were originally identified as network motifs in transcriptional networks, we also discuss why sRNAs may be used over protein transcription factors to help transduce environmental signals.

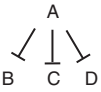
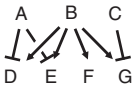
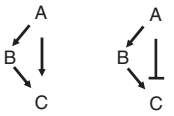
Introduction

Bacteria are programmed to effectively sense and respond to changes in their environment. The strength and nature of the response are often governed by complex regulatory networks encoded within the bacterial genome. These networks transduce environmental stimuli such as the presence of deleterious agents or changes in nutrient availability or temperature into finely tuned, global changes in gene expression. The changes allow the bacteria to resist chemical and biological threats and efficiently utilize available material and energy resources.

At the heart of regulatory networks are distinguishable patterns called regulatory circuits. These circuits are composed of nodes, which correspond to genes, and connecting lines, which correspond to regulatory interactions between each gene and its interacting partners (Table 1). Efforts to systematically identify regulatory circuits within the transcriptional regulatory architecture of different organisms led to the identification of network motifs, defined as common regulatory circuits that appear more often than expected by chance (Milo *et al.*, 2002; Shen-Orr *et al.*, 2002). Transcriptional network motifs were first identified in *Escherichia coli* and later found within the regulatory architecture of other organisms (reviewed in Seshasayee *et al.*, 2006; Alon, 2007). The identified motifs vary in size from one node, such as an autoregulated gene, to as many as a few hundred nodes, such as an array of cross-regulated genes. The regulatory impact of each motif varies. Some motifs accelerate gene activation or repression, while others amplify the response or reduce cell–cell variability in the expression of regulated genes. Still other complex motifs function as logic gates that determine whether the regulated genes are repressed or induced based on the combination of stimuli received.

Despite our extensive knowledge of transcriptional regulatory networks, little is known about how other types of regulators participate in regulatory networks. One class of regulators in bacteria that has gained recent attention is the regulatory small RNAs (sRNAs). Bacterial sRNAs are widespread and functionally diverse regulators with a predominant size range of 50–250 nucleotides (reviewed in Babitzke & Romeo, 2007; Wassarman, 2007; Waters & Storz, 2009). The most prevalent class of sRNAs relies on the RNA chaperone Hfq to form limited base-pairing interactions with target mRNAs. Base pairing between the sRNAs and the target mRNAs leads to changes in mRNA translation and stability, thereby influencing target gene expression. Ongoing characterization of this class of sRNAs has linked

Table 1. Summary of different regulatory circuits that incorporate sRNAs

Circuit name	Diagram	Benefits
Single-input module (SIM)		Coordinate activation or repression of multiple genes Introduce hierarchical ordering
Dense overlapping regulon (DOR)		Integrate multiple signals
Positive feedback (PF) loop		Slow down regulatory response Amplify signal
Negative feedback (NF) loop		Speed up regulatory response Reduce cell–cell variability Alter the relationship between inducing signal and regulated genes
Feedforward (FF) loop		Alter dynamics of target regulation Alter dynamic range Generate transient pulse in target regulation Integrate more than one signal

Arrows designate positive regulation and bars designate negative regulation.
A type-1 coherent loop (left) and type-1 incoherent loop (right) are shown for the feedforward loop.

Hfq-binding sRNAs to a broad range of environmental responses, including nutrient starvation, quorum sensing, membrane stress, oxidative stress, and many others.

Efforts to identify the regulators that control sRNA expression and the targets of Hfq-binding sRNAs have provided an initial list of interacting partners. By pinpointing where these interacting partners of sRNAs fall within regulatory architectures, we are gaining a clearer picture of how Hfq-binding RNAs are integrated into regulatory circuits. Understanding the behavior of these circuits is key to understanding the behavior of the encompassing pathways. In this review, we discuss the various regulatory circuits in which sRNAs are found and how these circuits contribute towards global regulation. Because many of these circuits were originally defined as network motifs in transcriptional regulatory networks, we also address why sRNAs may be used preferentially over protein regulators to transduce environmental stimuli.

The regulatory mechanism of Hfq-binding sRNAs

Before exploring the regulatory circuits that include Hfq-binding sRNAs, we will first discuss how Hfq-binding sRNAs regulate their targets. Hfq-binding sRNAs base pair with target mRNAs and modulate mRNA stability or translational efficiency (reviewed in Waters & Storz, 2009). Base pairing is mediated by the RNA chaperone Hfq, which generally binds A/U-rich stretches present in the sRNA and the target mRNA (Møller *et al.*, 2002; Zhang *et al.*, 2002; Soper & Woodson, 2008; Link *et al.*, 2009). Although a mechanistically defined role for Hfq is still under development, Hfq appears to serve a number of functions (reviewed in Valentin-Hansen *et al.*, 2004; Aiba, 2007; Brennan & Link, 2007). Hfq binding can alter the secondary structure of bound sRNAs and mRNAs, thereby promoting base pairing between the RNAs and increasing the rate of sRNA : mRNA association. For many unpaired sRNAs, Hfq binding also protects against degradation by the endoribonuclease RNAse E. In contrast, Hfq recruits RNAse E to initiate the degradation of some sRNA : mRNA pairs.

sRNAs have the capacity to repress or activate the expression of their target genes. Repressing sRNAs can inhibit translation, increase the rate of mRNA degradation, or both. Translational inhibition often results from the sRNA blocking recruitment of the ribosome by base pairing within the vicinity of the ribosome-binding site of the mRNA. In addition, sRNAs can bind upstream of the ribosome-binding site to inhibit translation by blocking ribosomal recruitment to a standby ribosome-binding site or an upstream ORF (Darfeuille *et al.*, 2007; Vecerek *et al.*, 2007). The resulting mRNA : sRNA complex often undergoes rapid degradation, although it is unclear whether this complex is a preferred target of RNAses or the reduced recruitment of

ribosomes leaves the complex vulnerable to RNAse attack. In one case of translational inhibition studied in detail, degradation of the mRNA : sRNA complex was not required for target repression and instead was proposed to facilitate the clearance of base-paired mRNAs (Maki *et al.*, 2008). However, sRNA-based repression can also occur through mRNA destabilization without affecting the rate of mRNA translation (Pfeiffer *et al.*, 2009). Thus, there are different mechanisms by which sRNAs can repress target gene expression.

The characterized examples of sRNA-based activation of gene expression involve similar mechanisms (Majdalani *et al.*, 1998; Majdalani *et al.*, 2002; Prevost *et al.*, 2007). The target mRNAs contain a stem–loop that sequesters the ribosome-binding site, thereby inhibiting translation. The sRNA base pairs with the stem–loop within the mRNA, releasing the ribosome-binding site and allowing ribosome binding and translation to occur. Importantly, some sRNAs such as RyhB and DsrA act as both activators and repressors (Majdalani *et al.*, 1998; Lease & Belfort, 2000; Massé & Gottesman, 2002; Prevost *et al.*, 2007).

Regulatory circuits involving sRNAs

Network motifs were originally delineated from a comprehensive set of the known transcription regulators and their regulatory targets for a specified organism. The elucidation of similar motifs for sRNAs will require an equally comprehensive set of sRNA targets and regulators, which is not yet available.

However, the regulators of the expression of many Hfq-binding sRNAs in *E. coli* have been identified. Typically, the regulator is a protein transcription factor capable of directly sensing a biological signal or a two-component system that responds to environmental stimuli. A wide range of environmental stimuli affect the expression of sRNAs, including anaerobic growth (Fnr activates FnrS), oxidative stress (OxyR activates OxyS), glucose availability (CRP represses Spot 42), iron availability (Fur represses RyhB), and osmotic imbalance (EnvZ–OmpR activates MicF) (Polayes *et al.*, 1988; Takayanagi *et al.*, 1991; Altuvia *et al.*, 1997; Massé & Gottesman, 2002; Boysen *et al.*, 2010; Durand & Storz, 2010). The levels of a few sRNAs are also regulated by competition with other RNAs. Known examples include GlmZ, whose deleterious processing is inhibited by the sRNA GlmY, and ChiX/MicM, which undergoes rapid degradation after base pairing with the *chiBCARFG* mRNA (Urban & Vogel, 2008; Figueroa-Bossi *et al.*, 2009; Overgaard *et al.*, 2009).

Identification of the gene targets of sRNAs has been more challenging. Microarray analysis following sRNA overexpression has been used predominantly, although this technique misses target genes solely regulated at the level of translation and cannot distinguish between direct and indirect targets of sRNAs (Altuvia *et al.*, 1997; Massé *et al.*, 2005; De Lay & Gottesman, 2009; Durand & Storz, 2010). Computational tools can predict base pairing between an sRNA and different mRNAs across an entire genome, although the limited number of base pairs observed between sRNAs and their known targets leads to high false-positive rates (Tjaden *et al.*, 2006). Despite these challenges, researchers have identified and validated an increasing number of targets of different sRNAs. While the set of gene targets is most likely incomplete for any given sRNA, this list provides a starting point to identify common regulatory circuits that incorporate sRNAs (Fig. 1). Many of these circuits were originally identified as network motifs within transcriptional regulatory networks.

Single-input module (SIM)

SIM is the simplest regulatory circuit that incorporates sRNAs and is one of the most common network motifs in transcriptional regulatory networks (Table 1). Here, a single regulator coordinately activates or represses the expression of multiple genes. Ideally, none of the target genes regulate each other. SIMs synchronize the expression of target genes in order to produce a coordinated response to a change in environmental conditions. SIMs can also establish a hierarchical order of regulation, where the time required to modulate gene expression is shorter for some target genes and longer for others. Hierarchical ordering has been observed in various cellular processes, including amino acid biosynthesis, chemotaxis, and cell cycle regulation (Laub *et al.*, 2000; Kalir *et al.*, 2001; Zaslaver *et al.*, 2004).

To date, most Hfq-binding sRNAs form SIMs by repressing multiple genes in response to a particular environmental stimulus. At first glance, the sRNA seems superfluous because it acts as a bridge between the environmental sensor controlling sRNA expression and the genes targeted by the sRNA. One explanation is that sRNAs often reverse the relationship between the environmental sensor and the sRNA targets: the repressor becomes an indirect activator and the activator becomes an indirect repressor. This general effect can be observed for sRNAs that regulate a few targets or many targets.

One example of an sRNA with few known targets that appears to reverse the effects of its regulator is SgrS. This sRNA is induced by the transcription regulator SgrR in response to phosphoglucose stress (Vanderpool & Gottesman, 2004). Once expressed, SgrS downregulates the expression of *ptsG*, which encodes a major glucose transporter. Interestingly, SgrS also encodes the small protein SgrT, which inhibits glucose transport (Wadler & Vanderpool, 2007). The cooperative functions of SgrS and SgrT allow SgrR to quickly and resolutely shut down glucose transport

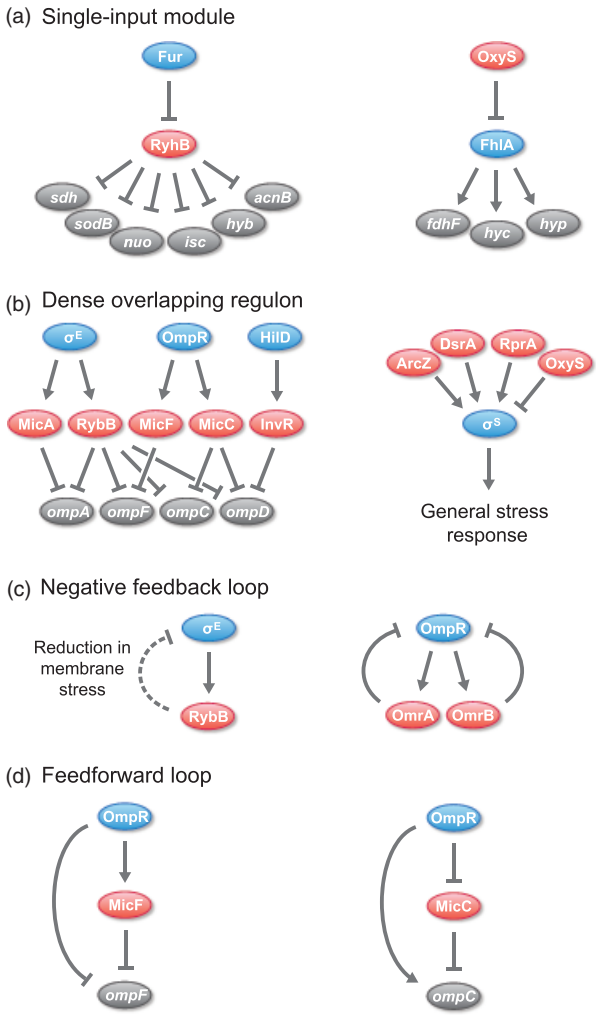


Fig. 1. Overview of regulatory circuits in which sRNAs are found in *Escherichia coli* and *Salmonella enterica*. The regulator controlling sRNA expression is sensitive to an environmental stimulus, such as the buildup of intracellular iron activating Fur or osmotic shock inducing the phosphorylation of OmpR by the cognate surface receptor EnvZ. (a) The single-input module (SIM) coordinates the expression of multiple genes through a single regulator. The sRNA incorporated into the SIM can directly target multiple genes or target a global regulator. (b) The dense overlapping regulon controls the expression of a common set of genes in response to multiple signals. Examples include the regulation of outer membrane proteins and the alternative sigma factor σ^S. (c) The negative feedback loop allows an sRNA to repress its own expression. Feedback can be direct or indirect depending on whether the sRNA targets its own regulator or relieves the stress responsible for activating sRNA expression. (d) The feedforward loop integrates two regulatory branches to control the expression of one target gene. The two known examples control the levels of two major outer membrane proteins through the combined regulation by OmpR and an OmpR-regulated sRNA. Arrows designate activation, bars designate repression, and dashed lines designate indirect regulation. Transcription regulators are in blue, sRNA regulators are in red, and target genes and operons are in gray.

when activated by phosphoglucose stress. Thus, this SIM converts SgrR from a transcription activator into an indirect repressor through SgrS and SgrT. Although SgrR represses its own expression independent of phosphoglucose stress (Vanderpool & Gottesman, 2007), there is no evidence that SgrR directly represses any other genes as part of the stress response.

A well-established example of an sRNA that regulates many targets is RyhB (Fig. 1a). RyhB is repressed by the transcription regulator Fur in response to high concentrations of intracellular iron. When iron is scarce, the derepressed RyhB downregulates at least 18 operons encoding iron-using proteins involved in diverse pathways, including the tricarboxylic acid cycle, dismutation of superoxide radicals, and iron storage (Massé *et al.*, 2005). Excluding the known targets of RyhB, the genes within the Fur regulon are generally repressed in the presence of excess iron. Thus, RyhB appears to switch Fur from a repressor to an indirect activator.

When targeting multiple genes, sRNAs may establish a hierarchical order of regulation (Levine *et al.*, 2007; Mitarai *et al.*, 2007). Hierarchical ordering was predicted through computational analyses, but has not been tested *in vivo*. The computational predictions hold that mRNAs with more extensive base pairing to the sRNA are regulated first, while mRNAs with less extensive base pairing to the sRNA are regulated last. Greater affinity between an sRNA and a set of mRNAs will force the sRNA to interact with these mRNAs over mRNAs capable of less extensive base pairing to the sRNA. Only when sRNAs have fully saturated the first set of higher-affinity mRNAs will excess sRNAs begin interacting with the second set of lower-affinity mRNAs.

If hierarchical ordering by sRNAs in SIMs occurs in cells, then there are two important ramifications. First, sRNAs could delay the regulation of particular target genes to provide an appropriate temporal response. Second, under a low or a transient environmental stimulus, only the expression of genes with extensive base pairing to the sRNA would be modulated. Therefore, target genes of lesser importance in the cellular response could be affected only after a strong and sustained stimulus. Examining the dynamics of target gene regulation by sRNAs in different cellular responses will help reveal whether sRNAs are capable of hierarchical ordering and how hierarchical ordering is utilized.

Finally, SIMs that incorporate sRNAs can indirectly influence global expression by controlling the expression of other regulators. In particular, mRNAs encoding transcription regulators appear to be common targets of sRNAs. In *Vibrio harveyi*, the Qrr family of sRNAs represses the synthesis of LuxR, the master regulator of quorum sensing (Tu & Bassler, 2007). In *E. coli*, the sRNA OxyS represses the synthesis of FhlA, a transcription activator of operons involved in formate metabolism (Fig. 1a) (Altuvia *et al.*,

1997; Altuvia *et al.*, 1998). In these examples, the sRNA acts as a signaling intermediate between the environmental sensor and the master transcription regulator. As described above for RyhB and SgrS, the Qrr family and OxyS RNAs also reverse the incident regulation: upregulation of Qrr expression by LuxO-P leads to LuxR repression and upregulation of OxyS expression by OxyR leads to FhlA repression.

It is worth noting that protein repressors can also switch the sign of regulation, introduce a regulatory hierarchy, and control the expression of global regulators. This begs the question as to why sRNAs would appear in SIMs instead of protein repressors. We return to this question later in the review.

Dense overlapping regulon (DOR)

The next type of regulatory circuit, the DOR, is also found throughout transcriptional regulatory networks. This circuit combines multiple overlapping SIMs (Table 1). Because each SIM responds to different environmental stimuli, the DOR coordinates the response to multiple biological signals. When multiple SIMs target a common set of genes, the encompassing DOR determines the relative expression of each target gene in response to different combinations of the detected signals. Thus, DORs may be important when controlling the expression of genes implicated in multiple biological responses.

Efforts to identify the target genes of sRNAs have revealed a common gene set regulated by multiple sRNAs, including the outer membrane proteins (Omps) and the alternative sigma factor σ^S (Fig. 1b). Many sRNAs modulate the expression of at least one Omp and a few, such as OmrA/B, RybB, and the MicA/C/F RNAs, target multiple Omps (reviewed in Vogel, 2009). One hypothesis for the prevalence of Omps as sRNA targets is that Omps play pivotal roles in cell survival: Omps act as the gatekeepers of small molecules entering and leaving the cell, serve as recognition elements for host immune responses and phage infection, and must be precisely regulated to preserve membrane integrity. Of the four major porins in *Salmonella enterica* (OmpA/C/D/F), all are targets of multiple sRNAs (Fig. 1b). The resulting network of sRNAs and targeted Omps forms a DOR that remodels the protein content of the outer membrane in response to different stress signals (reviewed in Vogel, 2009). This DOR responds to three known stress-related conditions associated with specific transcription regulators: extracytoplasmic membrane stress (σ^E), osmotic shock (OmpR), and host cell invasion (HilD). Extreme heat shock or a buildup of denatured proteins in the periplasm frees sequestered σ^E to activate the expression of the sRNAs RybB and MicA, which coordinately repress all of the major Omps. Osmotic shock activates MicF and represses MicC, which separately repress OmpF and OmpC/OmpD, respectively. Finally, host cell invasion activates InvR, which represses OmpD. These same sRNAs also target additional Omps, further expanding the size of this DOR.

Another example of an sRNA-based DOR converges on one target, the alternative sigma factor σ^S (Fig. 1b). This sigma factor acts as a master transcription regulator of the general stress response and is subject to multiple levels of regulation (reviewed in Hengge-Aronis, 2002). Within the extensive list of σ^S regulators in *E. coli* are four different sRNAs: ArcZ, RprA, DsrA, and OxyS. The first three sRNAs upregulate σ^S while OxyS downregulates σ^S. Of these sRNAs, ArcZ, RprA, and DsrA have been shown to directly base pair with the transcript encoding σ^S (Majdalani *et al.*, 1998; Majdalani *et al.*, 2002; Soper *et al.*, 2010). These three sRNAs activate the translation of σ^S by binding a region of the long 5′ UTR responsible for sequestering the ribosome-binding site. ArcZ, DsrA, RprA, and OxyS are expressed under stationary-phase growth, low temperature, envelope stress, and oxidative stress, respectively (Altuvia *et al.*, 1997; Majdalani *et al.*, 1998; Majdalani *et al.*, 2002; Papenfort *et al.*, 2009), suggesting that the common stress regulator σ^S integrates distinct stress signals with the help of these sRNAs.

The major benefit of DORs appears to be the coordinated regulation of a common set of genes in response to different biological signals, especially when the signals occur simultaneously. While the biological signals that activate the two DORs described above have only been evaluated in isolation, cells likely experience multiple stresses at the same time. Thus, it would be worthwhile to investigate how DORs specify the levels of the target genes in response to a combination of stress signals.

Feedback loop

The feedback loop offers a much simpler type of circuit in comparison with the large-scale DORs. This circuit occurs when a regulator controls the expression of its own gene, establishing a closed loop (Table 1). Regulation can be positive or negative depending on whether the loop enhances or dampens the sensitivity to a change in the biological signal. The search for network motifs in transcriptional architectures identified only autoregulatory loops, where the regulator directly controls its own expression (Thieffry *et al.*, 1998; Milo *et al.*, 2002). Thus, most of our knowledge surrounding the benefits of feedback loops comes from studies of autoregulatory loops composed of transcription activators or repressors. How these benefits change for feedback loops that incorporate multiple regulators is yet to be systematically established.

The characterization of positive and negative autoregulatory loops revealed opposing regulatory properties. Positive autoregulation can slow down the response time and

introduce variability in protein levels across the cell population (Maeda & Sano, 2006). Following the appearance of the inducing signal, more time is required to fully accumulate the protein regulator than in the absence of feedback because the levels of the regulator must build to a minimal concentration before its own transcription can further increase. In addition, cell–cell variability in protein levels is higher because a small increase in regulator levels is amplified by further activation of transcription.

In contrast, negative autoregulation can speed up the regulatory response (Rosenfeld *et al.*, 2002) and buffer against cell–cell variability (Becskei & Serrano, 2000). The response time is shorter than in the absence of feedback because an intermediate accumulation of the regulator leads to repression of its own transcription. Negative autoregulation also reduces cell–cell variability because a decrease in the levels of the regulator reduces transcriptional repression while an increase in the regulator levels improves repression. Negative autoregulation can also affect the relationship between the intensity of the inducing signal and the expression levels of the target genes (Yu *et al.*, 2008; Nevozhay *et al.*, 2009).

Hfq-binding sRNAs are unable to form autoregulatory feedback loops because they do not directly influence their own transcription. However, these sRNAs can participate in mixed feedback loops with transcription regulators. We define two types of loops that incorporate sRNAs: direct feedback loops and indirect feedback loops. Direct feedback loops involve the sRNA targeting its own regulator, while indirect feedback loops involve the sRNA affecting the activity or the expression of its regulator by targeting other genes. All known examples of feedback loops that incorporate sRNAs are negative feedback loops (NF loops).

One example of an sRNA involved in indirect negative feedback is RybB (Fig. 1c). RybB is activated by the envelope stress sigma factor σ^E as discussed above in one example of a DOR. Cell envelope stress frees sequestered σ^E, which activates the transcription of RybB. RybB in turn downregulates the expression of major membrane porins, thereby reducing the buildup of Omps that contributes to the envelope stress. With fewer Omps to instigate the cell envelope stress response, σ^E is sequestered and RybB transcription is reduced. Thus, as observed in both *E. coli* and *S. enterica*, the synthesis of RybB is reduced when the repression of its target genes relieves the stress that originally induced sRNA expression (Papenfort *et al.*, 2006; Thompson *et al.*, 2007).

sRNAs can also mediate direct feedback loops as exemplified by OmrA and OmrB (Fig. 1c). These seemingly redundant sRNAs repress the translation of their transcription activator OmpR along with a host of Omps (Guillier & Gottesman, 2006, 2008). While direct base pairing between OmrA and OmrB with the *ompR* mRNA has been validated, the role of this feedback loop has not been explored. The NF loop could limit OmpR levels as suggested previously (Guillier & Gottesman, 2006, 2008) or the loop could reduce cell–cell variability in OmpR levels or affect the relationship between the intensity of the inducing signal and the expression of OmpR-regulated genes.

The sRNA RyhB provides an example of an sRNA that mediates both direct and indirect negative feedback. Iron starvation induces RyhB, which represses multiple iron-using genes within the SIM described above. When RyhB represses these genes, free iron accumulates in the intracellular environment, allowing Fur to repress the transcription of RyhB (Massé *et al.*, 2005). More recently, RyhB was shown to inhibit the translation of an ORF upstream of *fur*, which leads to reduced Fur synthesis (Vecerek *et al.*, 2007). What remains to be seen are the relative contributions of the direct and indirect loops towards Fur regulatory activity.

Perhaps one of the most complex examples of sRNA-mediated negative feedback is provided by quorum-sensing networks in *Vibrio* species. Here, multiple feedback loops are involved in the transduction of signals through the quorum-sensing response. To adequately convey what is known about the sRNA-mediated feedback loops in this response, we first describe quorum sensing.

Quorum sensing is a form of chemical communication in which bacteria coordinate global gene expression based on cell density (reviewed in Ng & Bassler, 2009). Two distinct genetic and behavioral programs are established through quorum sensing: an asocial program at a low cell density characterized by a lack of coordinated behavior and a social program at a high cell density where cells may form biofilms, bioluminesce, or release virulence factors. In many bacterial systems including the marine bacterium *V. harveyi* and the human pathogen *Vibrio cholerae*, cell density is assessed according to the concentration of different autoinducer molecules secreted into the environment. Each molecule is detected through a cognate histidine kinase receptor of a two-component system. In *Vibrio*, when the autoinducer concentration is low (low cell density), receptors undergo autophosphorylation and transfer the phosphate group to the phosphotransfer protein LuxU. LuxU then transfers the phosphate group to the response regulator LuxO, yielding LuxO-P. LuxO-P in turn activates the transcription of multiple, homologous sRNAs (Qrr1-5 in *V. harveyi* and Qrr1-4 in *V. cholerae*). These Qrr RNAs then repress the synthesis of the quorum-sensing master regulator (LuxR in *V. harveyi* and HapR in *V. cholerae*). When the autoinducer concentration is high (high cell density), the autoinducer-bound receptors function as phosphatases and strip the phosphate from LuxO-P through LuxU. LuxO is unable to activate the *qrr* genes, resulting in the derepression of the master regulator.

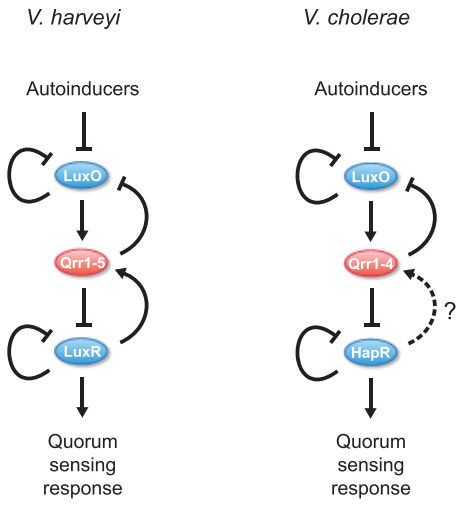


Fig. 2. Feedback loops in the *Vibrio* quorum-sensing phosphorelay cascade. In both *Vibrio harveyi* and *Vibrio cholerae*, in the absence of autoinducers, the histidine kinase two-component system phosphorylates LuxO to LuxO-P, which activates the expression of the Qrr family of sRNAs. In turn, the Qrr RNAs repress the expression of the quorum-sensing master regulator LuxR in *V. harveyi* and HapR in *V. cholerae*. A buildup of autoinducers leads to the deactivation of LuxO through dephosphorylation by the histidine kinase two-component system. Two of the identified feedback loops involve the Qrrs, while the other two involve autorepression by each transcription regulator. Autorepression by LuxO occurs independent of its phosphorylation state.

Recent studies dissecting the quorum-sensing phosphorelay circuit in *V. harveyi* and *V. cholerae* revealed multiple conserved feedback loops within the cascade (Fig. 2) (reviewed in Ng & Bassler, 2009). In *V. harveyi*, four feedback loops have been identified: LuxO represses its own transcription independent of its phosphorylation state, LuxR represses its own transcription, Qrr1-5 post-transcriptionally represses LuxO in addition to LuxR, and LuxR activates the transcription of Qrr2-4. The same loops are present in *V. cholerae*, only the LuxR homologue HapR induces the expression of the four Qrrs indirectly through an unknown mechanism.

To understand the roles of the feedback loops that include sRNAs, it is helpful to first picture how the quorum-sensing response changes between low and high cell densities. At a low cell density, cells continuously divide and secrete autoinducers that slowly accumulate in the environment. Once the bacteria reach a minimal density, the social genetic program must be enacted consistently and simultaneously across the population in order to maximize the group effect. In contrast, the transition from a high to a low cell density is rapid as cells are diluted or expelled into a new environment. Thus, cells transitioning from a low to a high cell density will experience a gradual increase in autoinducer concentration and yet require coordinated entry into the social mode, while cells transitioning from a high to a low density will undergo a sharp decrease in autoinducer and need to quickly exit the social mode in order to adapt to the new environment.

Insights into the contributions of the sRNA-mediated feedback loops to the quorum-sensing response were gained from the systematic disruption of each loop in *V. harveyi* and *V. cholerae* (Svenningsen *et al.*, 2008; Tu *et al.*, 2008; Tu *et al.*, 2010). In *V. harveyi*, the LuxO–Qrr feedback loop was disrupted by mutating the Qrr targeting sequences within the *luxO* gene (Tu *et al.*, 2010). This study demonstrated that the mutation increased the autoinducer concentration necessary to induce the expression of the LuxR transcription regulator and enter the social mode. This observation can be explained by the feedback loop reducing the LuxO pool available for phosphorylation in wild-type cells. By decreasing the size of the LuxO pool, less autoinducer is necessary to reduce the concentration of LuxO-P and induce the expression of LuxR. The LuxO–Qrr loop is thus proposed to tune the entry into the social mode to a lower cell density. Interestingly, disrupting this loop had no impact on the rate of LuxR accumulation or the cell–cell variability of LuxR. This suggests that these latter expected benefits of NF loops were masked by slower kinetics or larger cell–cell variability elsewhere in the quorum-sensing signaling cascade.

Another NF loop, LuxR/HapR activation of Qrr expression, was examined separately in *V. cholerae* and *V. harveyi* (Svenningsen *et al.*, 2008; Tu *et al.*, 2008). In *V. cholerae*, the HapR–Qrr loop was disrupted by deleting the *hapR* gene (Svenningsen *et al.*, 2008). Experiments where the autoinducer-saturated media were replaced with fresh media revealed an extreme lag in Qrr4 accumulation in the *hapR* deletion strain in comparison with the wild-type strain. The lag can be explained by the need for both LuxO-P and HapR for the rapid induction of the Qrrs. Upon the sudden loss of extracellular autoinducers in wild-type cells, LuxO-P accumulates before HapR protein levels diminish. Presumably, the HapR–Qrr feedback loop is also active during the transition from a low to a high cell density because LuxO-P and HapR would be simultaneously present under these conditions. However, disrupting the HapR–Qrr loop in *V. cholerae* had no impact on HapR expression in this transition. Therefore, in *V. cholerae*, the HapR–Qrr feedback loop only appears to be important in the transition from a high to a low cell density by accelerating the transition out of social mode.

A separate study in *V. harveyi* evaluated the same loop by mutating the LuxR operator site upstream of the *qrr* genes (Tu *et al.*, 2008). This study found that disrupting the LuxR–Qrr feedback loop increased the autoinducer concentration necessary to induce the expression of quorum-sensing genes. Here, more autoinducer is required in wild-type cells to overcome Qrr induction by LuxR and fully activate the expression of LuxR. This result suggests that the LuxR–Qrr loop in *V. harveyi* is important in the transition from a low to a high cell density and acts in opposition to

the LuxO–Qrr loop to tune the autoinducer concentration that initiates social behavior. As mentioned above, in *V. cholerae*, this feedback loop was not found to affect the transition into the social mode. While the discrepancy between *V. harveyi* and *V. cholerae* may suggest a difference in the quorum-sensing response, more work needs to be carried out to differentiate between these systems, devise a standard approach to evaluate the quorum-sensing response, and elucidate why some features of NF loops are masked in the transitions between a low and a high cell density.

Feedforward loop

The final type of circuit in which sRNAs have been identified, the feedforward loop, is a common transcriptional network motif in many organisms (Lee *et al.*, 2002; Milo *et al.*, 2002; Shen-Orr *et al.*, 2002; Boyer *et al.*, 2005). Feedforward loops are composed of two regulators controlling the expression of one gene (Table 1). The regulators can be sRNAs or proteins. For simplicity, we designate the regulators as A and B and the regulated gene as C. Regulator A controls the expression of genes B and C, and regulator B controls the expression of gene C. Thus, regulator A directly and indirectly controls the expression of gene C. Each regulatory connection can be positive or negative, yielding eight possible configurations of feedforward loops (Mangan & Alon, 2003). These eight configurations can be divided evenly into coherent and incoherent feedforward loops. Coherent feedforward loops feature both arms of the loop (A regulating C directly and through B) jointly activating or repressing C. For instance, in a type-1 coherent feedforward loop, A activates B and C, and B activates C (Table 1). Incoherent feedforward loops feature the arms acting in opposition. For instance, in a type-1 incoherent feedforward loop, A activates B and C, while B represses C (Table 1).

One computational study investigated how the loop configuration and the manner in which regulators A and B coregulate gene C impact loop function (Mangan & Alon, 2003). Results showed that both factors determine the overall dynamics of regulation. For instance, under AND logic (where both A and B are required to regulate C), the type-1 coherent feedforward loop introduces a time lag in the expression of gene C upon loop activation and filters out transient biological signals. Signal filtering may be important when cells should only respond to a sustained stimulus. Under OR logic (where either A or B is required to regulate C), the time lag occurs when the type-1 coherent feedforward loop is turned off. In contrast, the type-1 incoherent feedforward loop can generate transient pulses or accelerate the regulatory response. Accelerating the regulatory response with feedforward loops may be useful in various processes that necessitate a fast response, as suggested for sugar utilization (Mangan *et al.*, 2006).

The same computational study by Mangan & Alon (2003) also investigated how separate signals controlling the expression and activity of regulators A and B impact the expression of gene C. Results suggested that both signals can contribute to the regulation of gene C, although the relative extent depends on the specific configuration of the feedforward loop and how regulators A and B coordinately regulate gene C.

To date, only two feedforward loops that include sRNAs have been identified (Fig. 1d). Both loops are initiated by the response regulator OmpR to control the levels of the abundant porins OmpF and OmpC (Mizuno *et al.*, 1984; Chen *et al.*, 2004; Shimoni *et al.*, 2007). OmpR downregulates the expression of *ompF* and upregulates the expression of the sRNA MicF, which translationally represses *ompF*. In comparison, OmpR upregulates the expression of *ompC* and downregulates the expression of the sRNA MicC, which translationally represses *ompC*. Thus, OmpR participates in two coherent feedforward loops to repress the expression of *ompF* and upregulate the expression of *ompC*. Using the previous definitions of feedforward loop configurations, OmpR–MicF–OmpF forms a type-3 coherent feedforward loop (A represses C and activates B, and B represses C) and OmpR–MicC–OmpC forms a type-4 coherent feedforward loop (A activates C and represses B, and B represses C).

OmpR is activated through phosphorylation by the histidine kinase EnvZ in response to various environmental conditions, where increased osmolarity is the most well studied (reviewed in Pratt *et al.*, 1996). At low osmolarity, the low levels of OmpR-P activate the transcription of *ompF*. As osmolarity increases, higher levels of OmpR-P repress the expression of *ompF* and activate the expression of *ompC*. The regulation of sRNA expression by OmpR is less clear, although it appears that OmpR-P directly activates the expression of *micF* and either directly or indirectly represses the expression of *micC* (Takayanagi *et al.*, 1991; Chen *et al.*, 2004). Both MicF and MicC repress the translation of their target porin by base pairing with each target mRNA adjacent to the ribosome-binding site. Because each porin is expressed under opposing conditions, OmpR in combination with the sRNAs controls the ratio of OmpF and OmpC in the outer membrane: OmpF levels are high and OmpC levels are low under low osmolarity, while OmpC levels are high and OmpF levels are low under high osmolarity.

The ratio of OmpF and OmpC has been thought to be important in osmoadaptation by controlling the diffusion of small molecules across the outer membrane. The reasoning is that both OmpF and OmpC act as passive diffusion channels, where OmpF has a larger channel diameter and thus allows for greater diffusion across the membrane

(Nikaido & Rosenberg, 1983). Accordingly, cells would vary the ratio of OmpF and OmpC in the outer membrane to balance the uptake of nutrients without excessively accumulating environmental toxins.

Regardless of the true environmental stimuli that modulate the levels of OmpF and OmpC, both feedforward loops may be important in establishing the steady-state levels of these Omps, shaping the dynamics of the OmpR-mediated response, or integrating different environmental signals. Because both arms of the feedforward loops act together to regulate OmpF and OmpC, the inclusion of MicF and MicC in the feedforward loops could extend the dynamic range of the OmpF : OmpC ratio. The feedforward loops may also affect the adaptation time. One computational study showed that the type-3 coherent feedforward loop (such as OmpR–MicF–OmpF) is slower to activate gene C following loss of the detected signal as compared with direct regulation, whereas the type-4 coherent loop (such as OmpR–MicC–OmpC) does not introduce a time lag (Mangan & Alon, 2003). Whether the predicted lag following dephosphorylation of OmpR exists and is important in the adaptation process awaits evaluation. Finally, the expression of MicF and MicC is also modulated by temperature and growth phase (Chen *et al.*, 2004). By influencing the relative contribution of MicF and MicC to porin regulation, cells may tune the levels of OmpF and OmpC to match different environmental conditions.

It is noteworthy that the computational study that examined the dynamics of feedforward loops focused on transcriptional regulation (Mangan & Alon, 2003). A separate computational study recently examined the dynamics of feedforward loops that integrate sRNAs (Shimoni *et al.*, 2007). One portion of this study modeled the dynamics of the OmpR–MicF–OmpF loop and a transcriptional feedforward loop with the same configuration. While both feedforward loops introduced a lag in repression following the removal of the detected signal, the relative time scale of the lag depended on the sRNA degradation rate. The lag was relatively shorter for the sRNA-based feedforward loop if the sRNA underwent rapid degradation, whereas the lag was relatively shorter for the transcriptional feedforward loop if the sRNA underwent slow degradation. Thus, feedforward loops that integrate sRNAs may display altered dynamics in comparison with transcriptional feedforward loops. It will be interesting to see how RNA-based regulation determines the dynamics of other feedforward loops and how these dynamics shape cellular responses.

Why are sRNAs used instead of transcription regulators?

Hfq-binding sRNAs have been linked consistently to environmental responses in bacteria. As signal transduction could be conducted by other regulators including protein transcription factors, a broad question emerges: why are sRNAs used rather than protein regulators in these responses? A range of possibilities may explain the prevalence of sRNAs in particular regulatory circuits. These explanations include chance incorporation of sRNAs with no regulatory advantage, reduced metabolic cost, the need for additional layers of regulation, faster regulation, and the unique regulatory properties of sRNAs.

Equivalent regulators

A null hypothesis for the prevalence of Hfq-binding sRNAs is that these regulators offer no evolutionary advantage over transcription regulators. This hypothesis would predict that Hfq-binding sRNAs and transcription regulators are present in regulatory niches equally accommodating of either regulator. What would determine the relative frequency in any organism is how easily either regulator could fill a newly unoccupied niche. In both cases, key mutations must be accrued in the regulator or the target gene to establish a regulatory link. For many transcription regulators, the presence of short inverted repeat DNA sequences recognized by the regulator within the vicinity of the target gene promoter is sufficient. This link can emerge from mutations within the DNA-binding domain of the transcription regulator or around the target promoter.

For most examples of Hfq-binding sRNAs that negatively regulate gene expression, there are two known requirements to establish a regulatory link: Hfq binds the sRNA and target mRNA and the interaction between these RNAs forms a sufficient number of base pairs in the vicinity of the ribosome-binding site. According to a study of the base pairing requirements for the regulation of *ptsG* by SgrS, as few as six base pairs are critical for regulation (Kawamoto *et al.*, 2006). The requirements for gaining an Hfq-binding site are less clear; current data from *E. coli* suggest that this site must contain an A/U-rich stretch within the sRNA and the target mRNA (Møller *et al.*, 2002; Zhang *et al.*, 2002; Soper & Woodson, 2008; Link *et al.*, 2009). Sequencing of Hfq-bound mRNAs in *S. enterica* suggested that Hfq binds at least 1/5 of the transcribed mRNAs (Sittka *et al.*, 2008), although little is known about the number of cellular mRNAs bound by Hfq in other organisms. Further information about mRNAs bound by Hfq and the relationship between the extent of base pairing and target regulation will help explain how mRNAs become targets and whether new targets are more easily adopted by transcription regulators or sRNAs.

It is currently unclear whether protein transcription regulators and sRNAs can fill the same regulatory niche. Further efforts to elucidate regulatory pathways in different organisms may reveal examples of regulatory niches filled by the different types of regulators. In turn, these examples can

help us evaluate whether sRNAs confer any regulatory advantages in cells.

Reduced metabolic cost

Two potential advantages of sRNAs over protein regulators are inherent in their name: sRNAs are small and composed of RNA. Each sRNA gene occupies only a small section of the genome, limited energy is required to transcribe the ~100 nt sRNA, and no energy is expended to translate the sRNA into protein. In contrast, transcription regulators are encoded in larger pieces of DNA and must be translated from a much longer mRNA. The reduced energy consumption for the expression of sRNAs over protein transcription regulators leaves an energetic stockpile for cell growth and maintenance, thereby conferring a selective advantage. Reduced metabolic cost is one of the most repeated arguments for the prevalence of sRNAs (Mizuno *et al.*, 1984; Altuvia *et al.*, 1997; Massé & Gottesman, 2002).

However, when weighing the merits of this argument, additional contributors to the total metabolic cost must be considered. These contributors include transcription of the target gene and the relative levels of the sRNA and the mRNA encoding the transcription regulator. Pitting the costs of these contributors against the cost of translating the transcription regulator will help determine whether sRNA-based regulation is metabolically cheaper than transcriptional regulation. Based on the varied expression levels of target genes, sRNAs, and transcription factors, the relative metabolic cost may be sRNA-specific and even target gene-specific.

Additional layer of regulation

Another explanation for the prevalence of sRNAs reflects the limitations of transcriptional regulation in large-scale genetic networks. Transcription regulators typically control target genes by binding within a hundred nucleotides of the -35 and -10 promoter elements. On average, this stretch of sequence accommodates regulation by only a few transcription regulators. By targeting an entirely separate part of the gene, sRNAs expand the number of sites at which regulation can be introduced. Thus, sRNAs allow additional biological signals to control the expression of individual genes.

Layered regulation is especially important for genes that must be tightly controlled or are critical in multiple cellular responses. For instance, the alternative sigma factor σ^S is modulated under conditions ranging from stationary phase and cold shock to osmotic imbalance and low pH (Hengge-Aronis, 2002). Correspondingly, the levels of σ^S are regulated at the transcriptional, post-transcriptional, and post-translational levels. The four sRNAs shown to regulate σ^S, DsrA, RprA, ArcZ, and OxyS, are all induced under different stress conditions (Altuvia *et al.*, 1997; Majdalani *et al.*, 1998; Majdalani *et al.*, 2002; Papenfort *et al.*, 2009). One hypothesis is that σ^S came under regulation by sRNAs because additional regulation was difficult to evolve into a promoter already burdened with multiple regulator binding sites. Similarly, various sRNA targets such as *ompF*, *sdhC*, and *galK* are all subject to extensive transcriptional regulation (Weickert & Adhya, 1993; Pratt *et al.*, 1996; Park *et al.*, 1997).

An interesting observation related to transcriptional regulation is how the number of transcription regulators scales with genome size in bacteria. One bioinformatics study found that, in almost all bacteria examined, the number of transcription regulators scaled with the square of the total number of genes (van Nimwegen, 2003). In other words, bacteria with larger genomes used an increasingly larger fraction of transcription regulators. How do bacteria with larger genomes use these extra transcription regulators? Either individual genes are controlled by more transcription regulators or each transcription regulator on average controls fewer genes. By estimating the number of conserved sites upstream of bacterial promoters, a separate bioinformatics study concluded that the average number of binding sites was independent of genome size (Molina & van Nimwegen, 2008). Therefore, the transcriptional regulatory architecture in bacteria with larger genomes is anticipated to be more complex than in bacteria with smaller genomes.

These results raise the question as to how the number of base-pairing sRNAs and sRNA targets scale with genome size. Although sufficient data are not yet available to answer this question, the resulting correlation could reveal whether base-pairing sRNAs are important in the coordinated regulation of larger genomes or whether these sRNAs play a larger role in smaller genomes that have fewer transcription regulators on which to rely.

Faster regulation

Another possibility is that sRNAs have been selected over transcription regulators because of faster regulatory speed. Because sRNA-based regulation acts at the post-transcriptional level, gene expression is modulated at a point closer to protein production as compared with transcriptional regulation. Therefore, less time is required for an expressed sRNA to impact target protein levels. In support of this argument, a recent computational study evaluating the dynamics of different modes of regulation found that sRNAs achieved faster regulation than transcription regulators (Shimoni *et al.*, 2007). The improved speed was predicted when the expression of the regulator was both induced and repressed.

Why would it be useful for cells to speed up a regulatory response? Faster regulation may be beneficial in a highly

coordinated regulatory process or when cells must respond to a sudden change in environmental conditions. Given the close association between sRNAs and stress responses, the latter explanation seems plausible. Many of the environmental conditions that trigger sRNA induction require fast, yet global changes in expression. Placing sRNAs under the control of environmental sensors (i.e. OxyR, Fur, and CRP) or two-component systems (i.e. EnvZ–OmpR) would facilitate an expedited response by linking a post-translational modification of a response regulator (small molecule binding or protein phosphorylation) to induction or repression by an sRNA.

If cells can gain a selective advantage by rapidly responding to changes in environmental conditions, then why do environmental sensors not directly regulate genes targeted by sRNAs? Many environmental sensors, such as CRP or Fur, have extensive regulons independent of the regulated sRNA. Furthermore, some regulators such as the PhoQ/P or the EnvZ/OmpR two-component systems directly activate or repress many target genes within their regulons, potentially obviating the need for sRNAs. However, as discussed for SIMs, the direct targets of environmental sensors besides PhoQ/P and EnvZ/OmpR frequently are regulated opposite to the genes indirectly regulated through sRNAs. One explanation for the inclusion of sRNAs in regulatory networks is that some environmental sensors or their target promoters cannot easily evolve to allow gene induction or repression. By conscripting sRNAs, environmental regulators can more readily achieve both modes of regulation.

Conscription of a protein repressor could also allow the sign of regulation to be reversed. When fast responses are critical, the improved speed of sRNA-based repression might lead to the selection of sRNAs over protein repressors. The selection would be especially strong when regulating genes with highly stable mRNAs, as suggested for sRNA-based regulation of the Omps in *S. enterica* (Papenfort *et al.*, 2006). It would be informative to compare the regulatory dynamics of genes directly regulated by the environmental sensor and genes regulated by the sensor-controlled sRNA or protein repressor to determine how much the sRNA can accelerate the response dynamics and whether any improvement in speed would confer bacteria with a selective advantage in changing environments.

Unique regulatory properties

The final set of possible selective advantages of sRNA regulators comes from recent computational studies evaluating the quantitative characteristics of sRNA-based regulation (Levine *et al.*, 2007; Shimoni *et al.*, 2007; Legewie *et al.*, 2008; Levine & Hwa, 2008; Mehta *et al.*, 2008; Mitarai *et al.*, 2009). These studies constructed simple mathematical models to probe how regulatory performance is shaped by the different types of regulators: transcription regulators, which catalytically control the rate of transcription, and Hfq-binding sRNAs, which non-catalytically bind and regulate target mRNAs. Regulatory performance was determined by calculating the steady-state levels, cell–cell variability, and the dynamics of target gene expression. These studies predominantly compared repressors and negatively acting sRNAs, which are the focus of this section (Fig. 3), although some similar conclusions can be drawn for positively acting sRNAs.

Upon initial inspection, the response curves between protein-based and sRNA-based regulation appear to be significantly different, where each response curve relates the steady-state levels of the regulator and the target gene. Under protein-based regulation, target protein levels depend on the levels of the repressor protein and the affinity of the repressor for the target gene promoter (Fig. 3a). The resulting response curve displays the same general trend regardless of repressor affinity. Under sRNA-based regulation, target protein levels depend on the ratio of sRNA and mRNA production rates and the relative rate of sRNA action (Fig. 3c). When the rate of sRNA action is fast, the resulting curve follows a 'threshold-linear' response (Fig. 3c, red curve) (Levine *et al.*, 2007; Mehta *et al.*, 2008). The threshold-linear response can be divided into two regimes based on the relative sRNA and mRNA production rates. When sRNA production dominates (sRNA/mRNA production > 1), all mRNAs are bound and silenced. When mRNA production dominates (sRNA/mRNA production < 1), protein levels are reduced linearly with the rate of sRNA production. Thus, while protein-based regulation shows a graded response to repressor levels, sRNA-based regulation for a fast rate of sRNA action shows a two-regime response to the rate of sRNA and mRNA production with an ultrasensitive transition (sRNA/mRNA production ≈ 1) between regimes. However, recent work revealed that a slower rate of sRNA action changes the transition between regimes from sharp to graded (Fig. 3c, orange curve) (Mitarai *et al.*, 2009). Under a slower rate of sRNA action, there is less distinction between the response curves for sRNA-based regulation and protein-based regulation. Therefore, the benefits that can be derived from the threshold-linear response may be diminished depending on the parameters of sRNA-based regulation.

The differences in cell–cell variability, or gene noise, are more striking for genes regulated by proteins vs. sRNAs. Under protein-based regulation, when repressor levels are high, target protein levels are low and noise is amplified because of small-number effects from the transcription of a limited number of mRNAs (Fig. 3b). When repressor levels are reduced, target protein levels increase and noise is dampened as a greater number of transcribed mRNAs overcomes the small-number effect. In contrast, under sRNA-based regulation, noise is maximized at intermediate target

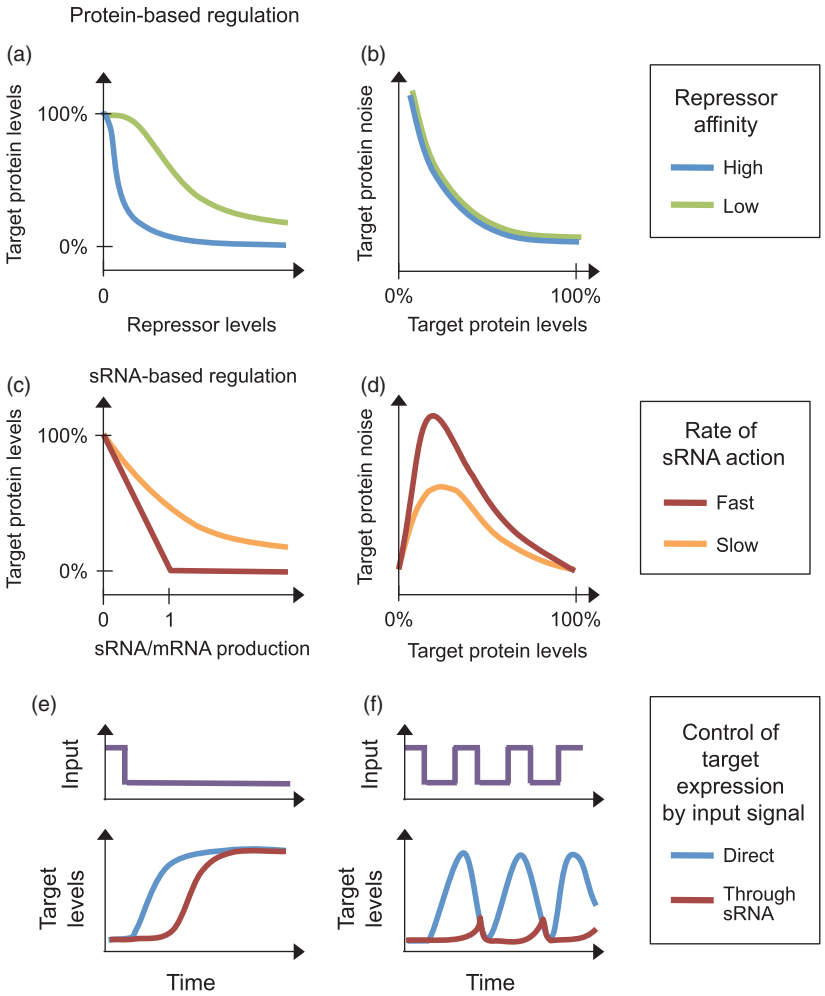


Fig. 3. The regulatory properties of sRNAs. (a) Response curve for a target gene negatively regulated by a protein repressor. Target protein levels depend on repressor levels and the affinity of the repressor for the target gene promoter. Target levels are more susceptible to high-affinity repressors (blue) than low-affinity repressors (green). (b) Noise profile for a gene negatively regulated by a protein repressor. Noise reflects the cell–cell variability in target protein levels across a bacterial population. The amount of noise decreases with increasing repressor levels and is generally independent of the affinity between the repressor and the target gene promoter. (c) Response curve for a gene negatively regulated by an sRNA. Target protein levels depend on the ratio of mRNA and sRNA production rates and the relative speed of sRNA action. When the speed of sRNA action is fast, the curve follows a 'threshold-linear' response (red). This response can be divided into two regimes based on whether sRNA or mRNA production dominates, with a sharp transition in between (sRNA/mRNA production = 1). When the speed of sRNA action is slow, the transition between regimes is graded (orange). (d) Noise profile for a gene negatively regulated by an sRNA. Noise is dampened when repressor levels are high because all transcribed mRNAs are bound by sRNAs and silenced. Noise is maximized in the transition between regimes because of ultrasensitivity near sRNA/mRNA production = 1. (e) Dynamics of target gene induction after the input signal is shut off for a protein repressor that directly senses the signal (blue) or for a negatively acting sRNA whose transcription is controlled by the signal (red). sRNA-based regulation introduces a time lag following increased transcription of the target gene or reduced production of the sRNA. (f) Dynamics of target gene regulation under a fluctuating input signal for a protein repressor directly sensing the signal (blue) or for a negatively acting sRNA whose transcription is controlled by the signal (red). sRNA-based regulation buffers against signal fluctuations due to the time lag when sRNA production is shut off. All axes are linear and each plot represents a pictorial representation of results from previous computational studies (Levine *et al.*, 2007; Legewie *et al.*, 2008; Levine & Hwa, 2008; Mehta *et al.*, 2008).

protein levels (Fig. 3d). When sRNA production dominates (sRNA/mRNA production > 1), target protein levels are low and noise is dampened as all mRNAs are bound and silenced. As the rate of mRNA production matches the rate of sRNA production (sRNA/mRNA production ≈ 1), noise builds and maximizes at the transition between regimes. Here, small fluctuations around the threshold yield either complete repression or the escape of a few mRNAs, magnifying the resulting noise in target protein levels. Increasing mRNA production into the linear regime (sRNA/mRNA production < 1) elevates target protein levels and gradually dampens noise to that achieved for high target protein levels

under protein-based regulation. Thus, sRNA-based regulation and protein-based regulation display different tendencies to amplify or dampen gene noise. The magnitude of gene noise can be beneficial or harmful to organisms, as high noise can disrupt the fidelity of cellular signaling and yet increase the phenotypic diversity in uncertain environments (Maheshri & O'Shea, 2007; Fraser & Kaern, 2009).

Unique aspects of the response curves and noise profiles have been used to argue for the prevalence of sRNAs. One of the most consistent arguments for sRNA-based regulation is the tight repression and low noise when sRNA production dominates (sRNA/mRNA production > 1). This regime limits the expression and cell–cell variability of genes and thus may be useful when regulating genes that are potentially harmful to cell viability or important in establishing the cellular phenotype.

The benefits of the linear regime (sRNA/mRNA production < 1) and the transition between regimes (sRNA/mRNA production ≈ 1) under sRNA-based regulation are less well established. Two studies suggested that the linear regime under sRNA-based regulation allows precise tuning of target gene expression levels (Levine *et al.*, 2007; Shimoni *et al.*, 2007), while another study suggested that sRNA-based regulation introduces additional noise as compared with protein-based regulation when tuning target levels (Mehta *et al.*, 2008).

The importance of the transition between regimes under sRNA-based regulation has also been under debate. Levine *et al.* (2007) argued that ultrasensitivity around the threshold (sRNA/mRNA production ≈ 1) amplifies the change in target gene expression from a small change in the intensity of the biological signal. However, Mehta *et al.* (2008) showed that the high noise in gene expression associated with changes around the threshold outweighs the contribution of ultrasensitivity. A rarely considered benefit of the transition between regimes is that heightened noise at the threshold may increase the phenotypic diversity in response to environmental stress. The relevance of this potential benefit needs to be further investigated.

As discussed above, regulation by sRNAs is predicted to be faster than regulation by protein repressors. However, despite the improved speed, there is the potential for a time lag between the appearance of a biological signal that reduces the production of the sRNA or increases the transcription of the target gene and the resulting change in target gene expression (Fig. 3e) (Legewie *et al.*, 2008; Levine & Hwa, 2008; Mitarai *et al.*, 2009). In either of these cases, existing sRNA molecules would need to be cleared before target mRNAs can accumulate. In comparison, direct regulation by an environmental sensor would rapidly alter the transcriptional rate of the target gene after a change in the environmental signal. However, if the environmental sensor controls the expression of a repressor protein, then genes targeted by the repressor would only be transcribed following degradation or dilution of the repressor. Therefore, both protein regulators and sRNAs are capable of generating a time lag when the expression of either regulator is controlled by the environmental sensor.

The lag associated with sRNA-based regulation displays two unique qualities over protein-based regulation. First, the lag only occurs when sRNA expression is turned off following loss of the biological signal. In comparison, a protein repressor whose transcription is controlled by the environmental sensor will display a lag when the biological signal appears (time to transcribe and translate repressor molecules) and is lost (time to dilute or degrade repressor molecules). Second, the time lag associated with sRNA-based regulation is more flexible. Coupled loss of paired sRNAs and mRNAs allows the rate of mRNA production to impact the length of the lag (Shimoni *et al.*, 2007; Levine & Hwa, 2008). Under protein-based regulation, the rate of mRNA production does not affect the turnover of the repressor, and therefore has no bearing on the time lag.

One potential advantage of a time lag is that it allows cells to differentiate between transient and sustained signals. If the biological signal has a shorter duration than the time lag, then the full cellular response will not be performed. In the case of a fluctuating signal, the time lag will effectively prevent induction under sRNA-based regulation (Fig. 3f). The ability to tune the time lag through sRNA-based regulation would allow cells to closely adjust the lag to different environmental conditions, leading to the induction of each response only when absolutely required.

Most sRNAs are induced under stress conditions. Because the time lag between the appearance of a biological signal and a change in target protein levels only occurs when sRNA production is reduced or the transcription of the target gene is increased, this lag would be observed predominantly in the transition from stressed to nonstressed conditions. This lag may or may not confer a benefit to cells. Cells often encounter repeated stress, where a rapid return to a nonstressed state may leave cells less competitive or vulnerable to further stress. Alternatively, the time lag may confer no competitive advantage and is instead a necessary evil of sRNA-based regulation. It would be helpful to investigate the dynamics of sRNA-based regulation into and out of inducing conditions across a wide range of cellular responses to determine how much the lag varies between different sRNAs and sRNA targets and whether the measured lag suits the particular environmental conditions.

Future directions

Identification of the interacting partners of sRNAs has revealed an assortment of regulatory circuits in which sRNAs are found. Whether any of these circuits represents

sRNA motifs – circuits that appear more often than one would expect by chance – remains to be evaluated. Once sRNA motifs are identified, a number of intriguing questions can be answered: what are the regulatory properties of sRNA motifs? How do sRNA motifs contribute to cellular signaling? How do the set of sRNA motifs compare with the set of transcriptional network motifs? How do the differences between these sets reflect the unique regulatory contributions of sRNAs in cells?

Before we can sufficiently answer these questions, the interacting partners of sRNAs and their direct regulators must be identified and validated thoroughly. In many instances, only a few of the potential sRNA targets have been confirmed as direct targets. By further identifying the direct targets of sRNAs, a more precise map of connections between sRNAs, their targets, and any associated transcription regulators can be constructed. From these maps, the network motifs that incorporate sRNAs can be extracted. The process of validating sRNA targets can be laborious, highlighting an opportunity for the development of high-throughput methodologies. Applying such methodologies outside of model organisms would considerably expand our knowledge of the prevalence of sRNAs and their targets in diverse bacterial species and allow us to address genomic questions originally reserved for transcription regulators: how do the number of sRNAs and sRNA targets scale with genomic size and how do the types and frequency of sRNAs vary based on natural habitat?

Recent computational studies have revealed various regulatory properties unique to sRNAs. These properties are predicted to impact the steady-state levels, dynamics, and cell–cell variability of sRNA target expression. While the debate continues on the relative importance of each property, it is worth noting that these properties were extracted from studies of sRNAs targeting individual genes or in SIMs. How these properties change in the context of regulatory pathways is only beginning to be investigated (Shimoni *et al.*, 2007; Levine & Hwa, 2008). One possibility is that many of the anticipated regulatory contributions of sRNAs to regulatory pathways are masked. As discussed above, the feedback loops formed by Qrrs in quorum sensing displayed few of the contributions associated with feedback loops (Tu *et al.*, 2010). However, it is also possible that sRNAs make unexpected contributions to entire signaling pathways.

Overall, we are only beginning to fully appreciate the roles that sRNAs play in bacterial regulation. These roles are closely tied to the function of large-scale regulatory pathways that help bacteria effectively sense and respond to their environment. Through continued efforts, we will gain a better understanding of how sRNAs are integrated into these networks and why sRNAs are used to mediate global regulation.

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